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HIV-1 persistence in CD4+ T cells with stem cell–like properties

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Cellular HIV-1 reservoirs that persist despite antiretroviral treatment are incompletely defined. We show that during suppressive antiretroviral therapy, CD4+ T memory stem cells (T_{SCM} cells) harbor high per-cell levels of HIV-1 DNA and make increasing contributions to the total viral CD4+ T cell reservoir over time. Moreover, we conducted phylogenetic studies that suggested long-term persistence of viral quasispecies in CD4+ T_{SCM} cells. Thus, HIV-1 may exploit the stem cell characteristics of cellular immune memory to promote long-term viral persistence.

Antiretroviral combination therapy effectively suppresses HIV-1 replication, but replication-competent virus can persist in memory CD4+ T cells despite treatment[1,2]. The memory CD4+ T cell compartment includes long-lasting central memory (T_{CM}) cells, which undergo a sequential developmental program with progressive commitment to more differentiated, short-lived effector memory (T_{EM}) and terminally differentiated T cell types[3,4]. The presence of a more immature memory T cell population with stem cell–like properties has previously been hypothesized on the basis of animal studies[5–9], and recently, small proportions of T cells with stem cell characteristics have been discovered in humans[10,11], mice[12] and nonhuman primates[13]. These cells, termed T_{SCM} cells, seem to represent the earliest and most long-lasting developmental stage of memory T cells, and can differentiate into large numbers of T_{CM}, T_{EM} and terminally differentiated T cells while maintaining their own pool size through homeostatic self-renewal. We hypothesized that HIV-1 can use CD4+ T_{SCM} cells as a preferred niche for promoting long-term viral persistence.

To test this concept, we initially investigated the susceptibility of CD4+ T_{SCM} cells to HIV-1 infection. These experiments demonstrated that CD4+ T_{SCM} cells, phenotypically defined as described in previous studies[10,14] and in **Supplementary Figure 1**, were approximately as susceptible as CD4+ T_{CM} cells to infection with an R5-tropic HIV-1 isolate (**Fig. 1a**), although their surface expression of CCR5 was slightly lower (**Supplementary Fig. 2a**,**b**). In addition, CD4+ T_{SCM} cells were highly susceptible to infection with a vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1 viral particles (**Fig. 1a** and **Supplementary Fig. 3**), despite their comparatively low expression of T cell activation makers (**Supplementary Fig. 4**). We also observed that HIV-1 RNA was readily detectable in CD4+ T_{SCM} cells from untreated HIV-1–infected patients (**Supplementary Fig. 2c**). CD4+ T_{SCM} cells had low sensitivity to the cytopathic effects associated with HIV-1 infection (**Supplementary Fig. 2d**) and expressed reduced levels of the cell-intrinsic HIV-1 restriction factors TRIM5α, APOBEC3G and SAMHD1 (**Supplementary Fig. 2e**). Together, these data indicate that CD4+ T_{SCM} cells are permissive to HIV-1 infection and can serve as physiologic target cells for HIV-1.

We next determined the levels of HIV-1 DNA in sorted CD4+ T_{SCM} cells from HIV-1–infected patients who had been treated with suppressive highly active antiretroviral therapy (HAART) for a median of 7 years (**Supplementary Table 1**). The proportions of CD4+ T_{SCM} cells in these patients did not differ from those in an HIV-1–negative control cohort (**Supplementary Fig. 5**). In these HAART-treated patients, per-cell levels of HIV-1 DNA were highest in CD4+ T_{SCM} cells, but their average contribution to the total viral reservoir in CD4+ T cells was only approximately 8% (**Fig. 1b**). Notably, in this cross-sectional analysis, the contribution of CD4+ T_{SCM} cells to the total viral reservoir in CD4+ T cells varied considerably among different HAART-treated patients and was inversely associated with HIV-1 DNA levels in the entire CD4+ T cell compartment (**Fig. 1c**). We observed this negative association selectively in the CD4+ T_{SCM} cell compartment (**Supplementary Fig. 6**), and it resulted in a disproportionately increased contribution of CD4+ T_{SCM} cells to the total viral CD4 T^+ cell reservoir in patients with a smaller viral reservoir in CD4+ T_{CM} and T_{EM} cells. This suggests that HIV-1–infected CD4+ T_{SCM} cells represent a not necessarily large but very stable and durable component of the viral CD4+ T cell reservoir that becomes increasingly prominent when viral reservoirs in alternative CD4+ T cell subsets are limited. HIV-1 DNA was also detectable in CD4+ T_{SCM} cells from elite controllers, a small group of HIV-1–infected individuals who maintain undetectable levels of HIV-1 replication in the absence of antiretroviral therapy[15], although at significantly lower levels than in CD4+ T_{SCM} cells from HAART-treated patients (**Supplementary Fig. 7**).

As only a small proportion of CD4+ T cell–associated HIV-1 DNA encodes replication-competent virus[16], we performed viral outgrowth assays from three study subjects who had been on continuous suppressive antiretroviral combination therapy with HAART for a median of 28 months (range 14–42 months). We were able to retrieve replication-competent virus from CD4+ T_{SCM} cells in all three cases, and the estimated frequency of cells harboring replication-competent HIV-1 in CD4+ T_{SCM} cells exceeded the corresponding frequencies in CD4+ T_{CM} and T_{EM} cells in two of the three patients (**Fig. 1d**). These findings

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indicate that HIV-1 DNA in CD4+ T_{SCM} cells is functionally capable of resuming active viral gene expression.

Given their stem cell-like properties, CD4+ T_{SCM} cells may represent a privileged site for long-term viral persistence. To better investigate this, we longitudinally analyzed HIV-1 DNA in sorted CD4+ T cell subsets from eight individuals who started antiretroviral therapy during primary infection and then remained on suppressive antiretroviral therapy without treatment interruptions. Using pair-wise comparisons between cell-associated HIV-1 DNA during earlier stages of antiretroviral therapy (median of 1 year, range: 10–14 months) and during later stages of treatment (median of 9 years, range 7–11 years), we observed stable or mildly decreasing viral DNA

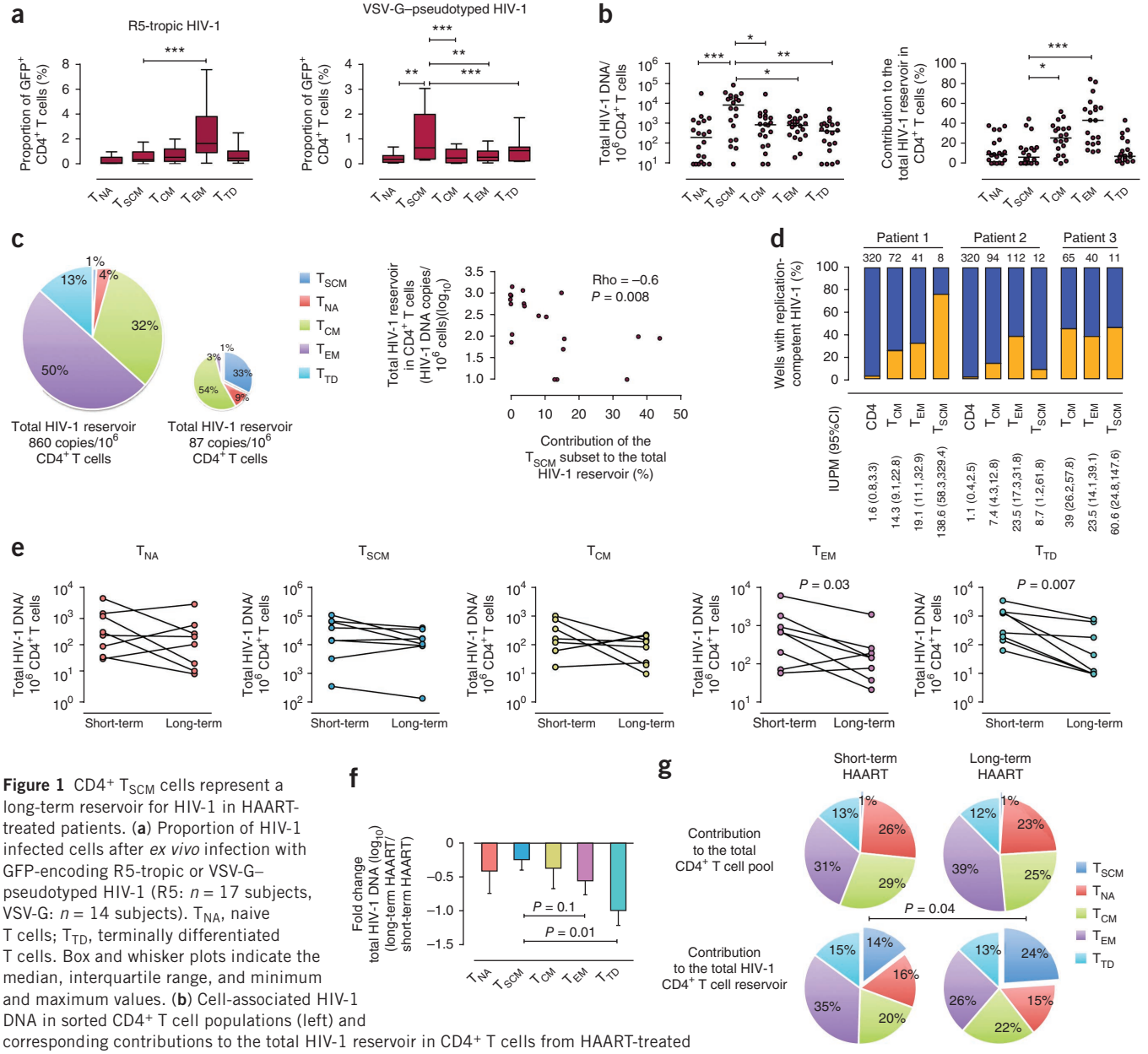

Figure 1 CD4+ T_{SCM} cells represent a long-term reservoir for HIV-1 in HAART-treated patients. (**a**) Proportion of HIV-1 infected cells after *ex vivo* infection with GFP-encoding R5-tropic or VSV-G–pseudotyped HIV-1 (R5: $n = 17$ subjects, VSV-G: $n = 14$ subjects). T_{NA}, naive T cells; T_{TD}, terminally differentiated T cells. Box and whisker plots indicate the median, interquartile range, and minimum and maximum values. (**b**) Cell-associated HIV-1 DNA in sorted CD4+ T cell populations (left) and corresponding contributions to the total HIV-1 reservoir in CD4+ T cells from HAART-treated individuals (right). Data from $n = 20$ subjects are shown; horizontal lines reflect the median. (**c**) Left, representative pie charts reflecting the contribution of CD4+ T_{SCM} cells to the total viral CD4+ T cell reservoir in two persons with large and small HIV-1 reservoirs in total CD4+ T cells, respectively. Right, Spearman correlation between contributions of CD4+ T_{SCM} cells to the total HIV-1 CD4+ T cell reservoir, and corresponding size of the HIV-1 reservoir in total CD4+ T cell from all patients shown in **b**. (**d**) Reactivation of replication-competent HIV-1 from memory CD4+ T cell subsets. Orange bars reflect proportions of wells with detectable replication-competent HIV-1; blue bars indicate proportions of wells without detectable replication-competent HIV-1. Numbers above columns reflect total numbers of wells analyzed for each CD4+ T cell population; numbers below columns reflect estimated frequencies of cells with replication-competent HIV-1 per million cells (IUPM) based on limiting-dilution analysis. CI, confidence interval. (**e**) Longitudinal evolution of HIV-1 DNA in CD4+ T cell subsets in $n = 8$ study persons who initiated antiretroviral therapy in primary infection. (**f**) Pair-wise fold differences in HIV-1 DNA levels measured after short-term and long-term antiretroviral therapy. Mean and s.e.m. from the eight study individuals from **e** are shown. (**g**) Corresponding contribution of individual CD4+ T cell subsets to the total CD4+ T cell pool and to the total HIV-1 CD4+ T cell HIV-1 reservoir after short-term and long-term antiretroviral therapy. Data are pooled from the eight study individuals from **e**. Statistical significance was tested with Wilcoxon's rank-sum test for panels **a**,**b**,**e**–**g**. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ after Bonferroni's correction for multiple comparisons in **a** and **b**.

levels in CD4+ T_{SCM} cells; the viral DNA decline in CD4+ T_{CM} and naive CD4+ T cells was slightly more pronounced (**Fig. 1e**). In contrast, in the more short-lived CD4+ T_{EM} and terminally differentiated T cell populations, we noticed a significant reduction in per-cell levels of total HIV-1 DNA over time (**Fig. 1e**). Notably, among all CD4+ T cell subsets, the relative longitudinal decline in total HIV-1 DNA at per-cell levels was smallest in CD4+ T_{SCM} cells, although differences between CD4+ T_{SCM} cells and naive CD4+T cells and T_{CM} cells did not reach statistical significance in our small study cohort (**Fig. 1f**). Of note, CD4+ T_{SCM} cells made a relatively small contribution to the total CD4+ T cell reservoir after the first year of suppressive antiretroviral therapy (**Fig. 1g**). Yet, after long-term antiretroviral treatment, there was a significant increase in the contribution of CD4+ T_{SCM} cells to the total viral reservoir in CD4+ T cells, despite the fact that the numeric contribution of CD4+ T_{SCM} cells to the total CD4+ T cell pool did not change. The contribution of CD4+ T_{CM} cells to the total viral CD4+ T cell reservoir also slightly increased over time, but this did not reach the level of statistical significance. In contrast, the contribution of CD4+ T_{EM} cells to the viral CD4+ T cell reservoir declined, despite a numerically increased proportion of T_{EM} cells in the total CD4+ T cell pool (**Fig. 1g**). These data, although collected from a limited number of patients, suggest that CD4+ T_{SCM} cells can support long-term viral persistence in patients treated with HAART.

We subsequently sequenced the proviral *Env* gene in DNA samples isolated from longitudinally sorted CD4+ T cell subsets from three HIV-1–infected patients who did not receive antiretroviral therapy during the initial years of disease, followed by continuous treatment

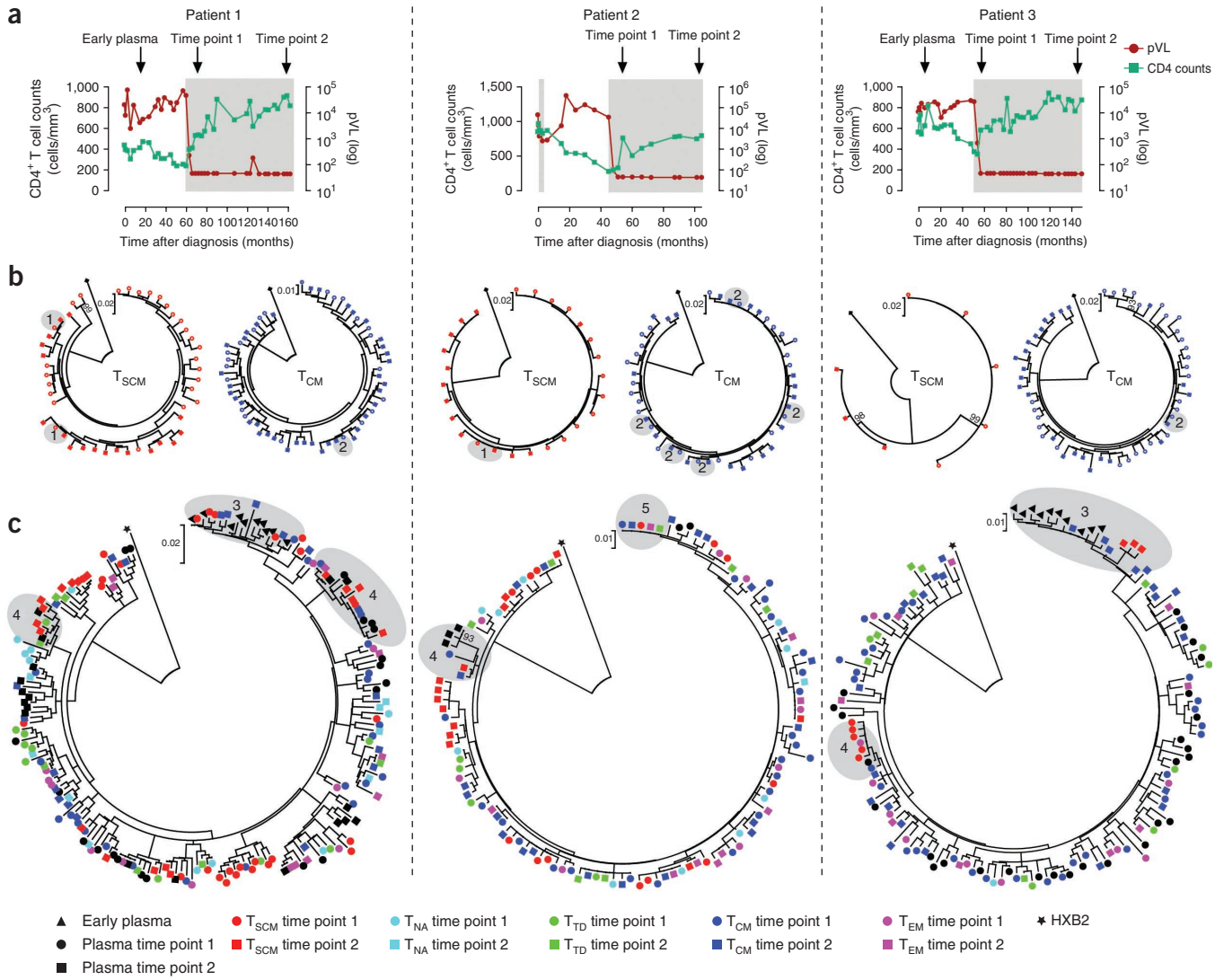

Figure 2 Phylogenetic analysis of HIV-1 sequences isolated from CD4+ T_{SCM} cells. (**a**) Longitudinal evolution of CD4+ T cell counts and viral loads in the three study patients described in the text. Shaded areas reflect periods of antiretroviral treatment exposure. Arrows indicate time of CD4+ T cell and plasma sampling. pVL, plasma HIV-1 viral load. (**b**) Phylogenetic analysis of HIV-1 sequences longitudinally amplified from sorted CD4+ T_{SCM} and T_{CM} cells at the beginning of antiretroviral treatment initiation and after 4–8 years of continuous suppressive therapy in three study persons. Identical HIV-1 sequences in T_{SCM} cells (1) and T_{CM} cells (2) are highlighted by gray circles. Circles represent sequences from time point 1; squares represent sequences from time point 2. (**c**) Circular phylogenetic trees of HIV-1 sequences amplified from the indicated CD4+ T cell subsets and from plasma collected at the time points indicated in **a**. Gray circles reflect phylogenetic relationships between HIV-1 DNA sequences from CD4+ T_{SCM} cells and circulating HIV-1 viral RNA sequences isolated during early untreated disease (3) or during contemporaneous and ensuing time points (4). Identical HIV-1 sequences isolated from CD4+ T_{SCM} cells, and from CD4+ T_{CM}, T_{EM}, terminally differentiated T cells isolated at later time points, are also highlighted (5).

with suppressive antiretroviral agents (**Fig. 2a**). We observed substantial intraindividual variability between viral sequences from CD4$^+$ T_{SCM} cells collected at the beginning of antiretroviral therapy and several years later, probably reflecting sampling of cells infected with different circulating viral strains during early disease stages (**Fig. 2b**). Yet, in CD4$^+$ T_{SCM} and CD4$^+$ T_{CM} cells (which were sampled in approximately 10- to 30-fold higher frequencies than CD4$^+$ T_{SCM} cells), we noticed several identical HIV-1 sequences in samples collected at the beginning of antiretroviral therapy and after 4–8 years of continuous treatment, which is consistent with long-term viral persistence in these CD4$^+$ T cell subsets (**Fig. 2b**). Notably, identical proviral sequences during early and later stages of antiretroviral therapy were not detected in naive or more terminally differentiated CD4$^+$ T cell subsets. We subsequently analyzed phylogenetic relationships between proviral HIV-1 *Env* sequences from CD4$^+$ T_{SCM} cells and circulating viral RNA amplified from plasma samples collected during early untreated disease stages and from residual HIV-1 viremia at the time of suppressive antiretroviral therapy. Notably, we observed that among all viral sequences from CD4$^+$ T cell subsets collected during suppressive antiretroviral therapy at later disease stages (6–12 years after infection), HIV-1 DNA isolated from CD4$^+$ T_{SCM} and T_{CM} cells was phylogenetically most closely related to circulating plasma sequences from early infection; this suggests that the viral strains circulating in early disease seem more likely to persist long term upon infection of CD4$^+$ T_{CM} and T_{SCM} cell subsets (**Fig. 2c**). In addition, pair-wise sequence comparisons revealed that the genetic distance between early plasma HIV-1 RNA sequences and HIV-1 DNA sequences from CD4$^+$ T cell subsets collected during later stages of infection was lowest for HIV-1 DNA sequences from CD4$^+$ T_{SCM} and CD4$^+$ T_{CM} cells (**Supplementary Fig. 8**). Sequences from CD4$^+$ T_{SCM} cells also showed phylogenetic associations with contemporaneous and ensuing sequences isolated from plasma during suppressive antiretroviral therapy, which is consistent with a possible interchange between viral strains in CD4$^+$ T_{SCM} cells and circulating viral species (**Fig. 2c**). Finally, we noted viral sequences from CD4$^+$ T_{SCM} cells at early stages of antiretroviral therapy that were identical to those from CD4$^+$ T_{CM}, T_{EM} and terminally differentiated T cells isolated several years later, supporting the role of CD4$^+$ T_{SCM} cells as precursor cells for more differentiated CD4$^+$ T cell subsets (**Fig. 2c**). Although these phylogenetic studies were performed in a limited number of patients, they suggest that CD4$^+$ T_{SCM} and T_{CM} cells may comprise a long-term reservoir for HIV-1.

This study indicates that CD4$^+$ T_{SCM} cells, despite their low frequencies, stand out among other memory CD4$^+$ T cell subsets as the cell population in which long-term HIV-1 persistence is particularly evident, probably owing to intrinsic cellular programs of these cells that give them superior abilities to self-renew, resist apoptosis and survive for extremely long periods of time[10,13]. Recently, stem cell–like functional properties were also observed in certain effector CD4$^+$ T cell subsets, such as T helper type 17 cells[17], and it will be important to analyze HIV-1 persistence in these long-lasting effector cell populations. Moreover, future studies will be necessary to determine whether a low viral reservoir in CD4$^+$ T_{SCM} cells represents a distinguishing feature of nonpathogenic simian immunodeficiency virus infection in natural hosts, as previously demonstrated for CD4$^+$ T_{CM} cells[18]. Interestingly, pharmaceutical inhibition of stem cell–specific molecular pathways is being investigated for targeting cancer stem cells[19], and the specific targeting of cellular pathways responsible for the stem cell–like properties of CD4$^+$ T_{SCM} cells may also have adjunct or additive effects on reducing the persistence of HIV-1–infected CD4$^+$ T_{SCM} cells. Thus, our increasing understanding of how stem cell–like properties of cellular immune memory maintain HIV-1 persistence despite HAART may be translatable into improved clinical strategies for inducing HIV-1 eradication and cure[20].

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.J.B., X.G.Y. and M.L. came up with the research idea, study design and concept. M.J.B. and M.L. wrote the manuscript. M.J.B., H.S., E.M.-G., J.L., J.Z.L. and T.J.H. performed experiments. M.J.B., X.G.Y. and M.L. analyzed and interpreted data. F.P., B.D.W. and E.S.R. contributed peripheral blood mononuclear cell samples from biorepositories. C.L., A.S. and K.S. provided technical assistance. R.Z. and Z.O. provided biostatistical assistance. J.Z.L., T.J.H., B.D.W., E.S.R. and X.G.Y. critically reviewed the manuscript. M.L. supervised all aspects of this study, including design, execution, interpretation and manuscript preparation.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Patients. Peripheral blood mononuclear cell (PBMC) samples from HIV-infected individuals or HIV-1–negative study subjects were used for this study according to protocols approved by the Institutional Review Board of Massachusetts General Hospital in Boston. All study participants gave written informed consent.

Cell sorting and flow cytometry. $CD4^+$ T_{SCM} cells and other $CD4^+$ T cell subsets were isolated according to a previously described protocol[14] with minor modifications. At least 100 million PBMCs were stained with monoclonal antibodies directed against CD4 (clone RPA-T4, BD Biosciences, 1:25 dilution), CD3 (clone UCHT1, Biolegend, 1:50 dilution), CD45RA (clone MEM-56, Life Tech, 1:50 dilution), CCR7 (clone 3D12, BD Biosciences, 1:25 dilution), CD62L (clone SK11, BD Biosciences, 1:25 dilution), CD122 (clone TU27, Biolegend, 1:25 dilution) and CD95 (clone DX2, BD Biosciences, 1:25 dilution). After 20 min, $CCR7^+CD45RA^+$ naive $CD4^+$ T cells, $CCR7^+CD45RA^-$central memory $CD4^+$ T cells, $CCR7^-CD45RA^-$ effector memory $CD4^+$T cells, $CCR7^-CD45RA^+$ terminally differentiated $CD4^+$ T cells and $CCR7^+CD45RA^+CD62L^+CD95^+C$ $D122^+CD4^+$ T memory stem cells were live-sorted in a specifically designated biosafety cabinet (Baker Hood), using a FACS Aria cell sorter (BD Biosciences) at 70 pounds per square inch. Cell sorting was performed by the Ragon Institute Imaging Core Facility at Massachusetts General Hospital and resulted in isolation of live lymphocytes with the defined phenotypic characteristics of >95% purity, as determined by three dedicated experiments in which sorted cells were subjected to repeat flow cytometric analysis (**Supplementary Fig. 1b**). For phenotypic characterization, cells were additionally stained with antibodies specific to CCR5 (clone 2D7/CCR5, BD Biosciences, 1:25 dilution) CXCR4 (clone 12G5, Biolegend, 1:25 dilution), CD38 (clone 90, Biolegend, 1:25 dilution) or HLA-DR (clone HIT2, Biolegend, 1:25 dilution) or annexin V and acquired on an LSRII flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Treestar).

Assessment of cell-associated HIV-1 DNA. Isolated $CD4^+$ T cells were digested as previously described[2] to extract cell lysates. We amplified total HIV-1 DNA with primers and probes previously described[21]. As a standard curve, we amplified serial dilutions of chronically infected 293T cells (kindly provided by F. Bushman). Proviral HIV-1 DNA copy numbers were calculated relative to *CCR5* gene copy numbers according to standard procedures.

Analysis of cell-associated HIV-1 RNA. Cell-associated HIV-1 RNA in sorted $CD4^+$ T cells was quantified by real-time RT-PCR, using primers and probes previously described[22]. HIV-1 RNA copy numbers were determined according to a standard HIV-1 RNA sample run in serial dilutions, and final results were expressed as the number of HIV-1 RNA copies per microgram of total RNA. The assay used had a detection threshold of 1 HIV-1 RNA copy per µg of total RNA.

Gene expression analysis. Expression of selected gene transcripts in individual $CD4^+$ T cell subsets was analyzed by semiquantitative RT-PCR using Taqman gene expression assays with standardized primers and probes and normalized to the expression of the housekeeping gene *Actb* (encoding β-actin) in each $CD4^+$ T cell subset.

***In vitro* HIV-1 infection assays.** Unselected PBMCs from HIV-1–negative donors were cultured in RPMI medium supplemented with 10% FCS and 50 U/ml of recombinant human interleukin-2 (rhIL-2). A total of 10×10^6 PBMCs were infected with a GFP-encoding, VSV-G–pseudotyped virus (multiplicity of infection (MOI) = 1, unless otherwise indicated) or a GFP-encoding R5-tropic viral strain (Ba-L, MOI = 1); both isolates were kindly provided by D. Littman. Cells were then washed twice with PBS and cultured at 200,000 cells/well in 96-well round-bottom plates for 5 d. On day 5, cells were stained with surface antibodies to identify individual $CD4^+$ T cell subsets, washed and analyzed on a LSRII flow cytometer.

Analysis of HIV-1 replication products. HIV-1 reverse transcripts were amplified from cell lysates with primers hRU5-F2 and hRU5-R and probe hRU5-P (early RT products) or with primers GagF1 and GagR1 and probe P-HUS-103 (late RT products)[23]. Integrated HIV-1 DNA was detected using nested PCR with Alu-1/Alu-2 primers and HIV-1 LTR primer L-M667 for the first-round PCR and LTR primer AA55M, Lambda T primers and MH603 probe for the second-round quantitative PCR, as described previously[24]. Serial dilutions of HIV-1 DNA from cell lysates of the HIV-1–infected cell line 293T (provided by F. Bushman, University of Pennsylvania) were used for reference purposes. Proviral HIV-1 DNA copy numbers were calculated relative to the *CCR5* gene previously quantified with the same standard curve. 2-LTR HIV-1 DNA was quantified as previously described[25].

Viral outgrowth assays. Sorted $CD4^+$ T cell populations were seeded at 10,000 cells/well (T_{SCM} cells) or 20,000 cells/well (T_{CM} and T_{EM} cells) in round-bottom 96-well plates. Subsequently, cells were stimulated with PHA (2µg/ml), rhIL-2 (100 units/ml) and irradiated allogeneic PBMCs from HIV-negative healthy donors. CD8-depleted, PHA-stimulated PBMCs from HIV-negative donors were added to each well on day 3 and again on day 7 and 14 of culture. Latently HIV-1–infected ACH-2 cells (NIH AIDS Reagent Program) were run as positive control cells, and $CD4^+$ cell–depleted PBMC samples from HAART-treated patients that were otherwise treated identically served as negative controls. The cultures were subjected to removal of 33% of the cell suspension every 7 d and replenished with fresh rhIL-2–containing medium. After 14–21 d, cell supernatant from each well was harvested, and the number of wells containing infectious HIV-1 was assessed by incubation of the supernatant with TZM-bl cells (NIH AIDS Reagent Program), a permissive HeLa cell clone that contains HIV-1 Tat-responsive reporter genes for firefly luciferase under control of the HIV-1 LTR, permitting sensitive and accurate measurements of infection. Luciferase activity was quantified by luminescence and is directly proportional to the number of infectious virus particles present in the initial inoculum. Estimated frequencies of cells with replication-competent HIV-1 were calculated using limiting-dilution analysis as described in ref. 26; all data were consistent with a single-hit Poisson distribution, as determined using a goodness-of-fit analysis based on a likelihood ratio test[26].

Viral sequencing. Cell lysates from sorted T cell populations and plasma were used for HIV-1 envelope sequencing encompassing the V3 region. For plasma samples, 6 mL of plasma from each time point were ultracentrifuged at 170,000*g* for 30 min before proteinase K digestion and RNA isolation by acid guanidiniumisothiocyanate. One-step RT-PCR reaction was then performed in triplicates using outer primers envA/LA11 (ref. 27). PCR products were used as a template to generate an amplicon by nested PCR with inner primers LA12 and LA13 (ref. 27). For V3 amplification from HIV-1 DNA in cell lysates, two-step nested PCR was performed with the same primer pairs. For amplification of HIV-1 RNA and DNA sequences, two to four separate reactions were conducted for each sample during first-round PCR; these PCR products were then pooled and used as templates for second-round PCR. Amplification products were inserted into TOPO cloning vectors and used to transform One Shot Stbl3 chemically competent *E. coli* (Life Technologies). Individual bacterial colonies were amplified by overnight culture, and extracted DNA was ligated and directly sequenced by T7 or T3 primers on an ABI 3100 PRISM automated sequencer, without prior PCR-based amplification. Sequences were aligned with an HXB2 reference sequence using BioEdit V7.1.9. A neighbor-joining method, as implemented in MEGA4 (ref. 28), was used to construct phylogenetic trees with phylogenetically informative HIV-1 nucleotide sequences. These sequences omit nucleotide mutations that occur only once and may therefore possibly be introduced by polymerase-induced errors during PCR[29]. Phylogenetically informative sites were identified as described before[29] (http://indra.mullins.microbiol.washington.edu/DIVEIN/insites.html). This conservative approach may slightly underestimate nucleotide diversity relative to single-template amplification methods, but a direct comparison between HIV-1 sequences derived by PCR/cloning and single-genome amplification in a number of our samples demonstrated equivalent population structure (**Supplementary Fig. 9**), consistent with prior studies[30]. For comparison purposes, viral sequences were analyzed by single-genome amplification according to a protocol described before[31].

Statistical analyses. Data are summarized as individual data plots with horizontal lines reflecting the median or as box and whisker plots indicating the median,

interquartile range, and minimum and maximum values. Spearman's correlation coefficient was calculated to analyze correlations. Differences were tested for statistical significance using Wilcoxon's rank-sum tests, Mann-Whitney *U* test, Kruskal-Wallis or Fisher's exact test, followed by Bonferroni's correction or Dunn's test for multiple comparisons where applicable.

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